Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2019; 8(2): 1306-1311
Received: 06-01-2019
Accepted: 09-02-2019

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In vitro antibacterial activities of crude ethanolic extract of *Vernonia amygdalina* (Asteraceae) and *Ocimum gratissimum* (Lamiaceae) and their combined effect on some bacterial isolates from sputum

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Abstract
Medicinal plants especially *Vernonia amygdalina* and *Ocimum gratissimum* are used as herbal remedies for infections and in some cases they are concocted together for their additive effect. This study, therefore investigated the antibacterial activity of the ethanolic leaf extract of both plants, alone and in combination on three microorganisms (*Escherichia coli, Klebsiella pneumoniae and Proteus vulgaris*) obtained from clinical isolate of sputum. The clinical isolates were identified and characterized using standard biochemical tests while the antibiotic sensitivity test was done using disc diffusion method. The ethanolic crude extract of *Vernonia amygdalina* and *Ocimum gratissimum* were also subjected to *in vitro* antibacterial test using Agar disc diffusion method while the combined antibacterial effect of both plants was done using agar strip diffusion method. Phytochemical tests using standard methods revealed the presence of flavonoids, alkaloids, saponins, tannins, cardiac glycosides and anthraquinones etc. Both of the plants had antibacterial effect which is represented by the inhibition zone diameter having a concentration-dependent relationship. At a concentration of 100 mg/ml, the highest inhibition zone diameter (13.66 ± 0.66 mm) and (12.86 ± 0.08 mm) were obtained for *V. amygdalina* and *O. gratissimum* respectively against *Escherichia coli.* The combined effect of the two plants however showed loss of antibacterial effect. The MIC of both plants were 25 mg/ml for *V. amygdalina* and *O. gratissimum* respectively. This study validates the use of the extracts of these plants for respiratory tract infection but their combined effect could be antagonistic and should therefore be discouraged because of loss of antibacterial activity.

Keywords: Vernonia amygdalina, *Ocimum gratissimum*, antibacterial, agar disc diffusion, minimum inhibition concentration

Introduction
Natural products including plants have always been a veritable source of medicine and nutraceuticals for treating various diseases and infections since antiquity [1, 2]. This is because of their innate ability to synthesize bioactive organic molecules [3]. Most people from poor and developing parts of the world still depend on plants (to about 80%), for a cheap, easily accessible, and efficacious source of medicine [4, 5]. Plants have a natural synthetic ability to produce bioactive, complex chemical structures that can oust the best synthetic chemist [6]. Furthermore, continued research into their bioactivity and chemistry have intensified with a motive to discover possible lead molecules which could translate into new antimicrobial compounds against the vast microbial resistance which has resulted to many treatment failures clinically [7, 8].

Medicinal plants have been used extensively in Nigeria and West Africa for their antimicrobial activities [9–11]. Among the plants used for herbal remedies are *Vernonia amygdalina* (Asteraceae) and *Ocimum gratissimum* (Lamiaceae). *Vernonia amygdalina* is a tropical shrub that can grow up to 3 m high. It is used traditionally (root, stem and leaves) to treat amoebic dysentery, stomach discomfort, kidney problems, fever, hiccups. anthelminthic and as an antimicrobial [12, 13]. Likewise the plant, *O. gratissimum* is used in traditional medicine as a laxative, stomachic, gargle for sore throats and tonsillitis. In Eastern Nigeria it is used for managing a newborn's umbilical cord. It is also used for upper respiratory tract infection, cough, fever, skin disease and conjunctivitis [14, 15]. Although, antibacterial activities of both plants has been undertaken on some selected organisms and clinical isolates, we hereby contribute to the studies by investigating their activities on clinical isolates from sputum of patients with upper respiratory infection.

In this study, therefore we investigated the antibacterial activity of *Vernonia amygdalina* and *Ocimum gratissimum* against some clinical isolates (*E. coli, K. pneumoniae, P. vulgaris*) obtained from patients sputum confirmed having respiratory tract infection.

Materials and Methods

Materials

The solvents used in this study were all of analytical grade (BDH). The different media such as Nutrient, Muller-Hinton and Cetrimide agar were all from -Titan Biotech Limited, India.

Collection of plant extracts

Healthy and matured leaves of Vernonia amygdalina (Asteraceae) and Ocimum gratissimum (Lamiaceae) were bought from the open market in Port Harcourt, Rivers State in the month of April, 2017. Botanical identification was done with herbarium voucher numbers UPH/V/1315 and UPH/V/1314 obtained for *Vernonia amygdalina* (Asteraceae) and *Ocimum gratissimum* (Lamiaceae) respectively.

Extraction of Plant material

The plant material was sorted out, air dried in a shade and finally pulverized. A sample (500g) of the powdered plant materials (*Vernonia amygdalina/Ocimum gratissimum*) were macerated differently for three days with absolute ethanol (BDH), filtered using Whatmann No 1 filter paper. The filtrate was concentrated in vacuum using Rotary evaporator at 30 ◦C. After complete evaporation of samples, the dried extracts were weighed and stored in sterile, air tight containers at 4 ◦C.

Preparation of Stock solution of crude extracts

A weight of the crude extracts (*Vernonia amygdalina* and *Ocimum gratissimum*) (1 g each) was reconstituted into 10 mL of 95% Ethanol respectively to obtain 100 mg/mL stock solution. Lower concentrations were prepared using double-fold serial dilution by transferring 5 mL of a particular concentration into 5 mL of 95% Ethanol to obtain: 50, 25, 12.5 and 6.25 mg/mL.

Isolation and chemical characterization of microorganisms

Preparation of Culture media

The following agar: Nutrient, Muller-Hinton, Cetrimide, MacConkey and Peptone water were prepared according to their manufacturer's specification. These agar plates were dried in a 37 ◦C incubator/ oven (Memmert ®, West Germany) and sterilized by autoclaving at 121 ◦C for 15 min.

Preparation of inoculum

The test organisms used were clinical isolates obtained from the laboratory of the Department of Medical Microbiology and Parasitology, University of Port Harcourt Teaching Hospital, Rivers State, Nigeria. The sputum was aseptically collected on sterile agar slants. The organisms were collected from the agar plates containing pure growth colonies by streaking using a wire loop in a zigzag pattern on the surface of a dried agar slant. This was to obtain actively growing cultures in their exponential growth phase. The plates were incubated (37 °C) for 24 h.

Gram staining test

The physical properties (color, smell and texture) of the microbial growth were noted. Gram staining test was conducted to identify and classify (Gram- positive or Gram-negative) each of the organism. The organisms were then sub-cultured on different media (Nutrient agar and MacConkey Agar as appropriate and incubated (37 °C) for 24h.The sub-cultured organism were isolated on a slant to obtain a pure culture and incubated ((37 °C) for 24 h.

Biochemical test

Conventional biochemical tests (Catalase, Triple sugar iron, Oxidase, Indole, Citrate and Gram staining tests) and morphological characterization were employed to identify the organisms.

Standardization of test organism

The isolated organisms (*Klebsiella pneumonia, Escherichia coli* and *Proteus vulgaris*), on agar slants were activated by sub-culturing into agar plates and incubating. Standardization was done by transferring each colony of organism using a sterilized wire loop into about 4 mL of Peptone water. The inoculum suspension was incubated at 35 ◦C to achieve a turbidity of 0.5 McFarland standard which is about 1.0×10^8 CFU/mL.

Antibiotic Sensitivity testing

The *in vitro* susceptibility testing of the isolated organisms were determined using Bauer- disc diffusion technique [16]. Commercial antibiotic disc by Potum Laboratories (India) were placed aseptically on 20 mL of Mueller Hinton agar plates inoculated with the test organisms according to the standard protocol described by National Committee of Clinical Laboratory Standards. The disc contained Ceftazidine (30 µg), Cefuroxime (30 µg), Gentamycin (10 µg), Ciprofloxacin (5 µg), Amoxicillin/ Clavunate (30 µg), Nitrofurantoin (300 µg), Ampicillin (10 µg).

Microbial susceptibility testing

Agar Disc Diffusion Method

The disc diffusion method [17, 18] was used to screen the plant extracts for antimicrobial activity. The Mueller Hinton Agar (MHA) plates were prepared by pouring 20 mL of molten media (already seeded with 0.1 mL of standardized inoculum) into sterile petri plates. The plates were allowed to solidify for 5 minutes. The concentrations of the extracts (100, 50, 25, 12.5 and 6.25 mg/disc) were loaded on 6 mm sterile disc. The loaded discs were then placed on the surface of the medium and the impregnated compound was allowed to diffuse into the medium for 5 minutes. The plates were kept for incubation at 37 ◦C for 24 hrs. At the end of incubation, inhibition zones formed around the disc were measured with a ruler in millimeter. These studies were performed in triplicates.

Antimicrobial activity of the plant extracts (*V. amygdalina* and *O. gratissimum* in a combined effect)

The Agar Strip Method

Adsorbent paper strips saturated differently with 12.5 mg/mL of each extract (*Vernonia amygdalina* and *Ocimum gratissimum*) were placed on the surface of the MHA plate already inoculated with the test organisms. The impregnated strips are placed so that they touch each other perpendicularly at one end. The plates were kept for incubation at 37 ◦C for 24 hrs. At the end of incubation, inhibition zones formed around the disc were measured with a ruler in millimeter. These studies were performed in triplicates.

Determination of Minimum inhibition concentration (MIC)

The agar dilution method [19, 20] was used to determine the MIC of the crude ethanolic extracts of both plants. Serial dilutions of the plant extract (100 - 6.25 mg/mL) in the sterile molten Mueller-Hinton agar media was prepared. The test organism was added to the various dilutions of the extract as a loopful of inoculum. The mixture was then poured into a sterile petri dishes and allowed to solidify. This was followed by incubation at 37 ◦C for 24 hrs. The plates were observed for microbial growth and the MIC was read as the least concentration that inhibited the growth of the test organism.

Phytochemical screening of the crude ethanolic extracts of the plants

The phytochemical screening for the crude ethanolic extracts of *Vernonia amygdalina* and *Ocimum gratissimum* were done according to standard methods [21, 22].

Result

The Biochemical Test Results

The bacterial samples were identified morphologically and hence classified into species using biochemical tests (Table 1). The presence of *K. pneumonia, E. coli and P. vulgaris* were confirmed by this study as the test organisms for the study.

Table 1: Identification of the Bacterial Species using Biochemical Tests

	Species of Clinical Pathogens		
Name of test	***Escherichia coli***	***Klebsiella pneumonia***	***Proteus vulgaris***
Oxidase	-	-	-
Catalase	+	+	+
Indole	+	-	+
Citrate	-	+	+
Triple Sugar Iron (TSI)			
Slope	Yellow	Yellow	Yellow
Butt	Yellow	Yellow	Yellow
Gas	Yes	Yes	No
H_2S	No	No	Yes
Motility	+	-	+
Gram- staining	Negative	Negative	Negative
Shape	Rod	Rod	Rod
Key: (-) = Absent; (+) = Present			

Standard Antibiotic sensitivity test

The clinical isolates from patient's sputum includes pathogenic organisms like *E. coli, K. pneumoniae* and *P. vulgaris* and they were very susceptible to all the antibiotics except to Ampicillin. This result indicates that the isolated organisms were not of a resistant strain as shown in Table 2.

Table 2: Inhibition zone diameter of antibacterial reference standards (disc) on the bacterial isolates from sputum

		Zone of inhibition (mm)		
Standard antibiotic	**Concentration (µg/ disc)**	***Escherichia coli***	***Klebsiella pneumonia***	***Proteus vulgaris***
Nitrofurantoin	300	24.00 ± 0.57	28.76 ± 1.39	26.21 ± 0.06
Amoxicillin/ Clavunate	30	-	10.97 ± 0.68	17.02 ± 0.42
Ofloxacin	5	23.40 ± 1.44	8.12 ± 0.40	5.04 ± 0.36
Ciprofloxacin	5	31.67 ± 1.45	10.11 ± 0.56	7.51 ± 0.79
Cefuroxime	3	13.84 ± 0.68	23.40 ± 1.51	13.93 ± 1.19
Ceftazidine	3	20.87 ± 1.45	24.45 ± 0.50	27.33 ± 0.88
Ampicillin	10	-	-	-
Gentamicin	10	23.11 ± 0.12	20.16 ± 0.61	19.38 ± 0.59
Values are mean of three replicates				

Microbial susceptibility testing using the plant extracts.

The two medicinal plants (*V. amygdalina* and *O. gratissimum*) showed varying degrees of antibacterial activity which is represented as the inhibition zone diameter (mm) against the three pathogenic organisms. The antibacterial activity also is shown to be concentration dependent (Tables 3 and 4).

Table 3: Inhibition zone diameter of the ethanolic extract of *Vernonia amygdalina* (bitter leaf) on the bacterial isolates from sputum

***V. amygdalina* extract**	**Test Organism**		
Concentration	**Zones of inhibition (mm)**		
(mg/disc)	***E. coli***	***K. pneumoniae***	***P. vulgaris***
100	13.66 ± 0.66	12.86 ± 0.08	10.66 ± 1.67
50	12.00 ± 2.90	10.33 ± 0.33	10.63 ± 0.88
25	11.66 ± 1.15	8.00 ± 0.88	8.00 ± 2.45
12.5	10.66 ± 2.02	7.33 ± 0.33	6.00 ± 1.90
6.25	8.33 ± 0.66	-	-
Key: (-) = growth or no zone of inhibition. Values are mean of three replicates			

Table 4: Inhibition zone diameter of the ethanolic extract of *Ocimum gratissimum* on the bacterial isolates from sputum

***O. gratissimum* extract**	**Test Organism**		
Concentration	**Zones of inhibition (mm)**		
(mg/disc)	***E. coli***	***K. pneumoniae***	***P. vulgaris***
100	10.66 ± 1.15	9.00 ± 0.08	-
50	10.00 ± 1.76	8.33 ± 0.23	-
25	8. 66 ± 0.57	8.00 ± 0.88	-
12.5	6.00 ± 0.87	7.73 ± 0.53	-
6.25	-	-	-
Key: (-) = growth of organism or no inhibition. Values are mean of three replicates			

Table 5: Inhibition zone diameter of the ethanolic extract of *Vernonia amygdalina* and *Ocimum gratissimum* (combined effect) on the bacterial isolates from sputum

V. amygdalina* and *O. gratissimum extract	**Test Organism**		
Concentration	**Zones of inhibition (mm)**		
(mg/ml)	***E. coli***	***K. pneumoniae***	***P. vulgaris***
100	-	-	-
50	-	-	-
25	-	-	-
12.5	-	-	-
6.25	-	-	-
Key: (-) = growth of organism or no inhibition and therefore antagonism			

Antimicrobial activity of the plant extracts (*V. amygdalina* and *O. gratissimum* in a combined effect)

The combined effect of the extracts (*V. amygdalina* and *O. gratissimum*) using agar strip method showed antagonistic effect (Table 5). The plants antibacterial effects nullified themselves which is represented by no inhibition zone diameter.

Minimum Inhibitory Concentration determination

The crude ethanolic extract of *V. amygdalin* and *O. gratissimum* plants (Table 6) showed minimum inhibitory concentration at 25 mg/ml.

Table 6: Minimum Inhibitory concentration (MIC) of the extracts of *V. amygdalina* and *O. gratissimum*

	Test Organism		
Plant species	***E. coli***	***K. pneumoniae***	***P. vulgaris***
	MIC (mg/ml)		
V. amygdalina	25	25	25
O. gratissimum	25	25	-
Key: (-) = no activity			

Phytochemical Analysis

The phytochemical screening of both plants (Table 7) shows the presence of different classes of phytochemicals such as alkaloids, tannins, anthraquinones etc.

Table 7: The phytochemical analysis of the ethanolic leaf extracts of Vernonia amygdalina (bitter leaf) and *Ocimum gratissimum*

Phytochemical Test	***Vernonia amygdalina***	***Ocimum gratissimum***
Alkaloids test		
Meyer's test	+	+
Hager's test	+	+
Dragendorff's	+	+
Tannins	+	+
Saponins test		
Frothing test	+	+
Emulsion test	+	+
Flavonoid test	+	+
Anthraquinones test	+	+
Carbohydrate test	+	+
Molish test	+	+
Fehlings test	+	+
Cardiac glycosides test	+	+
Terpenes test	+	+
Key: + = Present, (-) =Absent		

Discussion

The plants *Vernonia amygdalina* (bitter leaf) and *Ocimum gratissimum* (clove basil) have wide traditional usage as herbal remedy and their efficacy as antimicrobial, immune booster, hypoglycemic, antioxidant and antimalarial have been the subject of much scientific discourse [23–26]. In the present study, *in vitro* antibacterial activities of *Vernonia amygdalina* and *Ocimum gratissimum* were investigated against three clinical isolates from patient's sputum. The pathogenic bacteria (*E. coli, K. pneumoniae* and *P. vulgaris*) isolated from the patient's sputum represents the common microorganisms implicated in most respiratory tract infection in Nigeria [27, 28]. The antibiotic sensitivity test (Table 2) conducted on these organisms was to investigate resistance pattern of these organisms to the commonly prescribed therapy and their sensitivity results therefore qualified their use for this study. While the standard antibiotics such as the Ciprofloxacin®, Ceftazidine® and Nitrofurantoin® had good antibacterial activity on the organisms (Table 2), the Penicillins (Ampicillin® and Amoxicillin/ Clavunate®) had none indicating the existence of resistance. These results are however in agreement to a previous report of antibiotic resistance pattern of some bacteria infecting the respiratory tract in Nigeria [27]. The antibacterial activity of *V. amygdalina* and *O. gratissimum* were promising when used singly because at a concentration of 100 mg/ml, the zones of inhibition observed for *E. coli* were 13.66 ± 0.66 mm and 10.66 ± 1.15 respectively. There was concentration dependence in the antibacterial activity of both plants in the study. The plant extracts showed no antibacterial activity when used together because their mechanisms of antibacterial activity could be opposing to each other. The retardation in diffusion and permeation of the crude plant extracts through the growth media further contributed to the reduction in zones of inhibition because of poor solubility of the extract in the reconstituting solvent (95% ethanol). Better results may be expected from a universal solvent such as dimethyl sulfoxide (DMSO) which may overcome the solubility and permeation problems [29]. Drug resistance could be due to a fortified peptidoglycan cell wall, active drug-efflux pump mechanisms, alteration of enzymes or receptor site and production of biofilms by microorganism [30] that hinders the activity of antibacterial compounds. The organism *P. vulgaris* displayed a resistance to *O. gratissimum* which could have been as a result of the mechanisms mentioned above. The MIC of *V. amygdalina* against the three bacterial organisms (*Escherichia coli, Klebsiella pneumoniae and Proteus vulgaris*) were 25 mg/ml in this study, which differs from the results obtained in

1999 by Akinpelu [31]. In that study six of the tested organisms showed sensitivity to *V. amygdalina* at a concentration of 25 mg/ml but the MIC ranged from 3.13 – 6.25 mg/ml for the different organisms. The differing results in activity observed in these studies may be due to differences in the methods of extraction, origin and time of plant harvest, source of organisms and the methods adopted for the studies and handling. The MIC for *Ocimum gratissimum* was also 25 mg/ml for *Escherichia coli, Klebsiella pneumoniae* but it had no activity against *Proteus vulgaris.* This result does not agree with the result obtained in a study by Omodamiro [32] in which the MIC was reported to be 62.25 mg/ml. Secondary metabolites (tannins, flavonoids, alkaloids, anthraquinones and saponins) were abundantly present in both plant extracts and these compounds have been reported to possess medicinal property [33] and are therefore responsible for this antibacterial activity

Conclusion: The study has shown that ethanol crude extract of the leaves of *Vernonia amygdalina* and *Ocimum gratissimum* had antibacterial activities. This gives a scientific justification to its usage in the management of some respiratory tract infection.

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